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Symposium article

Targeting of cytokine gene expression to malignant melanoma cells using tissue specific promoter sequences

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Summary

Background: Transduction of tumour cells in vitro with cDNAs encoding various cytokines and/or immune accessory molecules has been shown to diminish or eliminate tumorigenicity when such cells are returned in vivo to syngeneic animals. One method being explored for in situ gene therapy is to use tissue-specific promoters to direct expression of the therapeutic genes to the tumour cells.

Design: This study used the 5' flanking region of the murine tyrosinase gene to direct expression of three different cytokine genes [murine interleukin 2 (IL-2), IL-4 and macrophage colony-stimulating factor (M-CSF)] specifically to murine melanoma cells.

Results: Expression of the IL-2 gene, from 2.5 kbp of the 5' flanking region of the murine tyrosinase gene, was detected in 11 out of 55 puromycinresistant B16 clones isolated after transfection. The highest producing clone secreted 2000 pg/ml/10^6 cells/48 hours as determined by enzyme-linked immunosorbent assay. The IL-2 was tested for biological activity by its ability to sumulate proliferation of the IL-2 dependent CTLL cell line. No detectable level of IL-2 expression occurred in 58 clones of drug-resistant NIH 3T3 cells derived after transfection with the same construct. Similar results were obtained following transfection of these two cell lines with the tyrosinase-IL-4 minigene construct. Expression of IL-2 in the murine melanoma cells completely abrogated their tumorigenicity in syngeneic mice. However, progressively growing tumours were produced from clones in which the IL-2 gene was no longer expressed (as determined by reverse transcriptase polymerase chain reaction). Direct injection of DNA encoding cytokine genes, expressed from the tyrosinase promoter, into established B16 melanomas in syngeneic mice resulted in gene expression within the tumour mass. While no change in tumour growth was observed following such treatment, the results demonstrate that direct injection of naked DNA into a neoplasm can result in uptake and expression of cytokine genes up to 16 days post-injection.

Conclusion: The use of tissue-specific promoters can limit expression to the required target cell, while the choice of appropriate gene should result in an alteration in tumour burden.

Key words: cytokines, gene therapy, tissue expression

Introduction

Transduction of tumour cells in vitro with cDNAs encoding various cytokines and/or immune accessory molecules has been shown to diminish or eliminate tumorigenicity when such cells are returned in vivo to syngeneic animals [1]. These results have fuelled the development of numerous vaccination protocols, which often suffer from the technically demanding and time-consuming requirements of having to remove cells from the patient to manipulate them in vitro [1]. Clearly, the in situ genetic modification of neoplastic cells provides an attractive and, by contrast, simpler approach to novel molecular therapies. However, such in situ gene therapy would require a specificity of gene delivery that is impossible using currently available viral vectors or physical transfer techniques [2, 3]. One way to circumvent these limitations would be to use tumour-specific promoters to direct expression of the therapeutic gene [4, 5]. Uptake of the inserted DNA by 'innocent' bystander cells could then be tolerated, as highly specific expression would occur only in neoplastic cells.

Melanin synthesis (a frequently observed characteristic of malignant melanomas) is regulated in a tissue-specific fashion partly because of melanocyte-specific transcription of the gene encoding tyrosinase, which is the key regulatory enzyme of this pathway [6, 7]. The 5' flanking region of this gene has been shown to direct expression of heterologous genes both in human and murine melanocytes and melanoma cells, while not permitting expression in a range of other cell types [5, 8]. Furthermore, we have shown that the direct intratumoural injection of naked DNA, containing either the Herpes simplex virus thymidine kinase gene or a reporter gene, driven by the tyrosinase promoter, can lead to a significant level of gene expression in melanoma cells [5].

The study reported here now demonstrates that cytokine cDNAs, when expressed in a weakly immunogenic murine melanoma, under the control of the tyro-

sinase promoter, reduce in vivo tumorigenicity of transfected cells. Injection of these constructs into established tumours resulted in efficient expression of these cytokine genes and (though alterations in growth rates were not observed), our results suggest that direct genetic modification may be a feasible therapeutic approach for patients with advanced melanoma.

Materials and methods

Construction of expression plasmids

Standard recombinant techniques were used for subcloning [9]. Restriction endonuclease enzymes were obtained from Northumbria Biologicals (NBL, Cramlington, U.K.), and Taq polymerase was supplied by HT Biotechnology Ltd (Cambridge, U.K.). Oligonucleotides, synthesized on an Applied Biosystems 380B synthesizer and purified by denaturing acrylamide electrophoresis, were provided by the Oligonucleotide Synthesis Laboratory (ICRF Clare Hall, South Mimms, U.K.). Polymerase chain reaction (PCR) amplification of DNA fragments was performed using a Techne PHC-2 Thermocycler, and reaction mixes were prepared in a hood isolated from normal areas of DNA handling. Amplified DNA sequences were subcloned into the PCR II vector (Invitrogen; British Biotechnology Products Ltd, Oxford, U.K.) and their identities were confirmed by restriction endonuclease mapping. The correct fragments were then shuttled from PCR II into the appropriate expression plasmid. Exact restriction maps and details of each construct are available on request.

The murine IL-2 gene (532 bp) was excised from the expression vector pBCMGNeo-mIL-2 [10] (a generous gift from Dr P. Frost). The murine IL-4 gene (450 bp) was obtained from British Biotechnology Ltd (Abingdon, U.K.), and the murine M-CSF gene (1.8 kbp) [11] was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.).

Cell culture

The B16 melanoma and NIH 3T3 fibroblast cell lines used in this study were checked routinely and found to be free of mycoplasma infection. Lines were grown in Eagle's minimal essential medium supplemented with 10% (v/v) foetal calf serum and 4 mM L-glutamine. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO_2.

Construction of the Tyr-(cytokine) expression plasmids (Fig. 1)

A 2494 bp fragment of the murine tyrosinase promoter (−46 to −2540) was cloned by PCR into the promoterless mammalian β-galactosidase (β-Gal) expression vector pNASS-β (Clontech, Palo Alto, CA, U.S.A.) to produce the plasmid Tyr-β-Gal-1 [5]. The murine IL-2 gene was cloned by PCR, with added NotI restriction sites, and ligated into Tyr-β-Gal-1 by replacing the β-Gal gene with the Il-2 gene to give Tyr-IL-2. In this plasmid, a 2.5 kbp fragment of the promoter region is used to express the murine IL-2 gene. Tyr-IL-4 and Tyr-M-CSF were generated by replacing the β-Gal gene with the murine IL-4 or murine M-CSF genes, respectively.

DNA transfection

A total of 10^6 adherent cells in monolayer culture were transfected with 10 μg of plasmid DNA by calcium phosphate co-precipitation using the Profection method (Promega, Madison, WI, U.S.A.). Twenty-four hours after the addition of the precipitate, cells were washed three times in serum-free medium and incubated in normal medium for 72–96 hours prior to being split into the selection medium [1.25 μg/ml puromycin (Sigma, Poole, U.K.)].

In vitro detection and quantitation of IL-2 and IL-4 production

Cells of the appropriate clone (5×10^5) were seeded in normal medium. Forty-eight hours later, supernatant was harvested and filtered (0.2 μm Nalgene filter) and the cells were trypsinized and counted. The supernatant was assayed for IL-2 by its ability to stimulate the proliferation of the IL-2-dependent T cell line, CTLL.

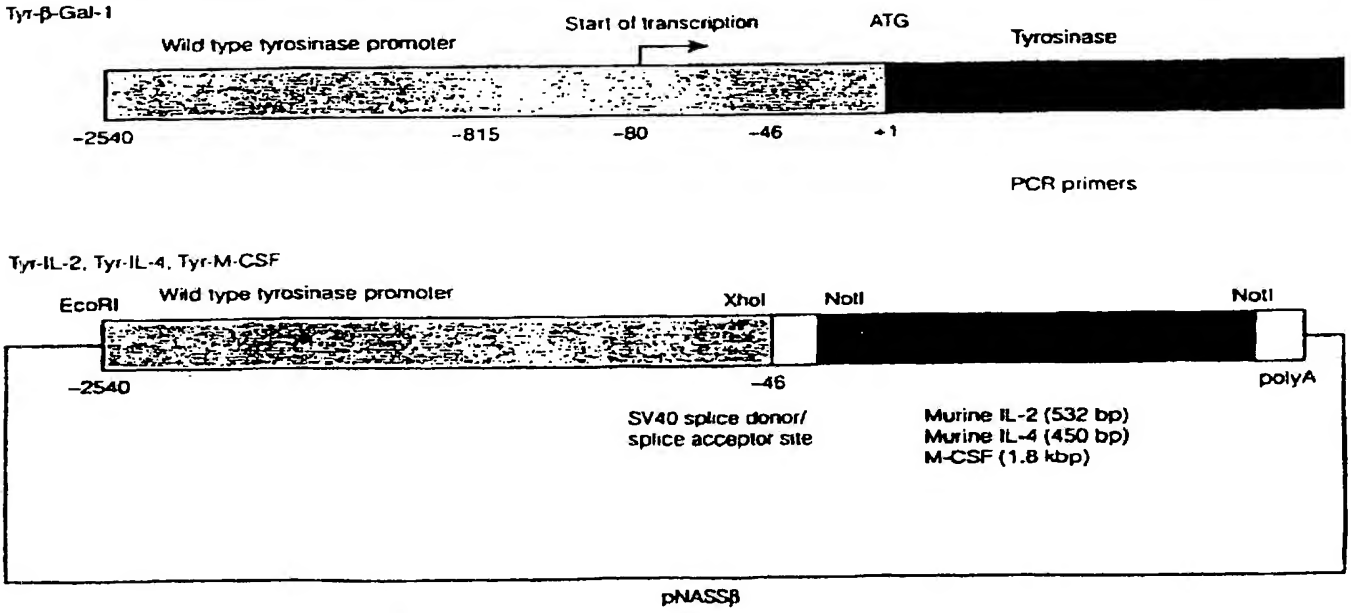

Fig. 1. The Tyr-(cytokine) expression plasmids.

Cells were grown in the presence of cell culture supernatants, standard concentrations of recombinant IL-2 (1.3, 2.5 or 5 ng/ml) or, as negative controls, tissue culture medium or supernatant from untransfected B16 cells. The rate of proliferation was measured by ^{3}H-thymidine incorporation after 4 days' growth [12, 13]. A similar bioassay was used to assess IL-4 production from transfected clones using the murine T-helper cell line, HT-2; murine recombinant IL-4 (British Biotechnology, Abingdon, U.K.) was used to calibrate the assay as described previously [14]. The results obtained from the CTLL bioassay for IL-2 production were confirmed by ELISA (Genzyme, Boston, MA, U.S.A.).

In vivo injection of cells

To assess tumorigenicity, 1×10^5 cells from selected clones were injected (100 µl inoculum volume) subcutaneously into the flank in groups of syngeneic mice. Animals were monitored daily until the tumour became palpable, after which the diameter, in two directions, was measured daily with calipers until it reached approximately 1.5 × 1.5 cm. Tumour volumes were calculated according to the formula:

$$\text{Volume} = (a^2 \times b)/a$$

where a and b are the minor and major dimensions, respectively.

Intratumoural injection of DNA

Progressively growing B16 melanomas, established as described above, were monitored until they were approximately 4 mm in diameter. Animals were anaesthetized halothane induction (ICI Pharmaceuticals, Macclesfield, U.K.) and the tumour was injected with 1 µg of the appropriate DNA as a calcium phosphate precipitate in 100 µl volume via a 27 gauge needle.

Detection of expression of cytokine genes in vivo by reverse transcriptase PCR

Tumour samples removed from animals which had received intratumoral injections of DNA were rapidly frozen following excision to ensure conservation of the RNA. RNA was prepared by homogenization of the tissue with RNAzol (Biogenesis Ltd, Bournemouth, U.K.) followed by RNA extraction. RNA concentrations were estimated by agarose gel electrophoresis, and 1 µg total cellular RNA was reverse transcribed in a 20 µl volume using oligo-(dT) as a primer and Moloney murine leukaemia virus reverse transcriptase (Pharmacia LKB Biotechnology, Milton Keynes, U.K.). A cDNA equivalent of 1 ng RNA was amplified by reverse transcriptase PCR (rt-PCR) in a 50 µl reaction mixture with 250 µM of each dNTP, 100 nM of primers, 5 µl of 10x buffer (HT Biotechnology Ltd, Cambridge, U.K.) and 1 unit of Super Taq DNA polymerase (HT Biotechnology Ltd, Cambridge, U.K.) using 40 cycles (94°C, 1 minute denaturation; 60°C, 1 minute, 30 seconds annealing; and 72°C, 2 minutes extension). The reaction mix (25 µl sample) was analyzed by agarose gel electrophoresis (1.2%) in TAE buffer containing 0.2 µg/ml ethidium bromide. In all experiments, a mock PCR (without cDNA) was performed to exclude contamination.

Results

In vitro specificity of IL-2 gene expression

The Tyr-IL-2 construct (10 µg) shown in Fig. 1 was co-transfected along with pSV2puro (0.5 µg) into either B16 melanoma cells or NIH 3T3 fibroblasts. Expression of the IL-2 gene was monitored in stable drug-resistant clones which had been isolated and expanded in 1.25 µg/ml puromycin. Of 55 B16 clones, 11 produced detectable levels of IL-2 (Fig. 2a), with the highest producing clone (clone 30) producing 2000 pg IL-2/ml supernatant/10^6 cells/48 hours. None of 58 clones of similarly selected NIH 3T3 fibroblasts produced any more than very low levels of IL-2. Assessment of functional IL-2 levels by stimulated proliferation of the CTLL-2 cell line, titrated against a standard curve of recombinant murine IL-2 (Genzyme), demonstrated good concordance between these two techniques to estimate the levels of secreted IL-2 (Fig. 2b). The data confirm the cell type specificity of the tyrosinase promoter for melanoma cells.

Similar results were produced by transfection of the Tyr-IL-4 plasmid into B16 and NIH 3T3 cells. Five pooled populations of puromycin-selected NIH 3T3 cells cotransfected with Tyr-IL-4 produced only minimal levels of IL-4 as determined by the HT-2 cell bioassay. In contrast, nine of 23 B16 clones were isolated

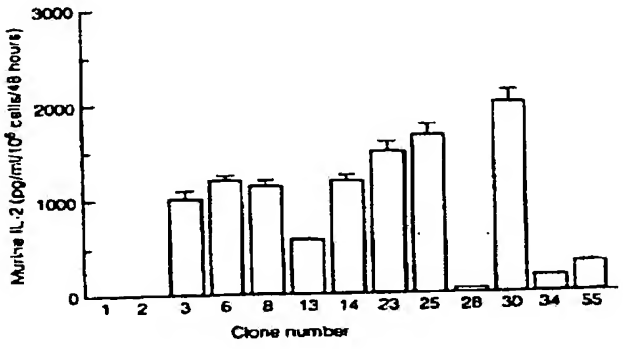

Fig. 2a. Production of murine IL-2 by B16 clones transfected with the Tyr-IL-2 expression plasmid as assayed by ELISA.

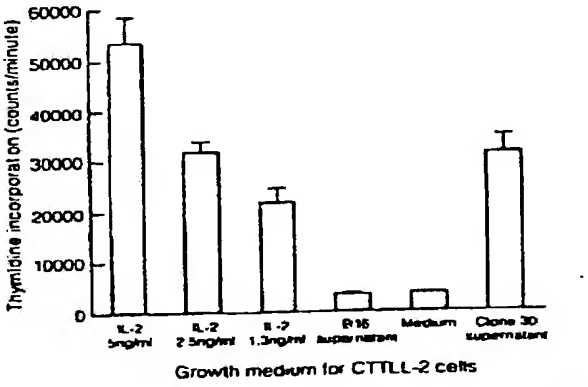

Fig. 2b. Stimulation of IL-2 dependent T cell line, CTLL-2, by standard concentrations of recombinant IL-2, untransfected B16 cell supernatant, tissue culture medium or the supernatant of clone 30.

which produced high levels of IL-4, with the best clone producing 750 pg IL-4/ml/10^6 cells/48 hours.

In vivo tumorigenicity of IL-2-producing clones

The tumour growth curves of clone 30 (high-level IL-2 producer), clone 3 (intermediate-level) and clone 28 (low-level IL-2 producer) and the control puromycin-resistant clone, B16-puro (B16 transfected with pSVPuro, no IL-2 production) are documented in Fig. 3. Whereas the control clone produced rapidly growing tumours in all animals (achieving a volume of 3 cm^3 24 days after injection), most of the IL-2 secreting clones failed to develop into tumours.

However, two of five animals injected with cells from clone 3 developed tumours whose growth rate, after an initial lag phase, paralleled that of the control cells (Fig. 3a). The remaining three animals remained free of tumours. The two tumours originating from injection of clone 3 cells were recovered and used to prepare RNA. When analyzed for expression of the IL-2 gene using rt-PCR, no expression could be demonstrated (Fig. 3b), whereas IL-2 message was detected in positive control samples.

These results indicate that loss of tumorigenicity correlates well with continued IL-2 expression. Such instability of expression was not seen under culture conditions, where stable levels of expression were maintained for over 4 months from these cells.

Direct intratumoural injection of Tyr-IL-2, Tyr-IL-4 and Tyr-M-CSF plasmid DNA

Established subcutaneous B16 melanomas in the flanks of C57 mice were injected with 1 μg of DNA. Tumours were injected either with individual cytokine-expressing plasmid alone (Tyr-IL-2 or Tyr-IL-4) or with a combination of three plasmids (0.3 μg each of Tyr-IL-2, Tyr-IL-4 and Tyr-M-CSF). A negative control consisted of 1 μg of control plasmid, PRO, in which the cytokine gene is absent (tyrosinase promoter alone). All DNA samples for injection were prepared as calcium phosphate precipitates.

Subsequent to DNA injection, tumour growth curves were determined and are illustrated in Fig. 4a. No statistically significant reduction in tumour growth was seen following injection of any of these cytokine expression plasmids either alone or in combination at the dose tried. However, using rt-PCR to monitor levels of cytokine mRNA, all three cDNAs were expressed in vivo up to 16 days after the single DNA injection (Figs. 4b and 4c).

To assess the sensitivity of the reaction in detecting cytokine gene expression, rt-PCR was performed on RNA prepared from 10^6 cells grown in vitro comprising different proportions of B16 cells and clone 28 cells (low-level IL-2 producers). The intensities of the bands detected from the tumour samples injected with either Tyr-IL-2 or Tyr-IL-4 alone were similar to those from a mixture of cells containing approximately 10^5 cytokine-expressing cells (Figs. 4b and 4c). This demonstrates that roughly 1% of the tumour cells are expressing the cytokine genes as long as 16 days after DNA injection, a value consistent with our earlier studies using a reporter gene [5]. In addition, expression of the genes appeared to be dose dependent; tumours injected with 0.3 μg of Tyr-IL-2 or Tyr-IL-4 gave a band of reduced intensity compared to tumours which were injected with 1 μg of the respective DNAs.

The possibility that rt-PCR was detecting residual injected DNA was excluded by using primer pairs in which the 5' primer recognizes an untranscribed region

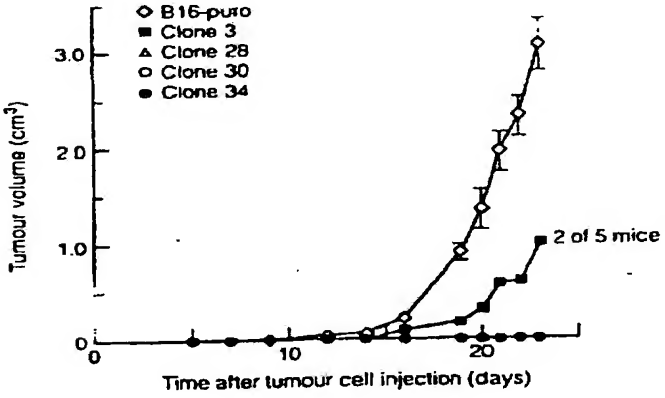

Fig. 3a. In vivo growth of IL-2 transfected B16 clones; 1×10^5 cells of each clone were injected subcutaneously into the flank of syngeneic mice and the tumour volumes measured at daily intervals. The values for clone 28, 30 and 34 overlap along the X-axis.

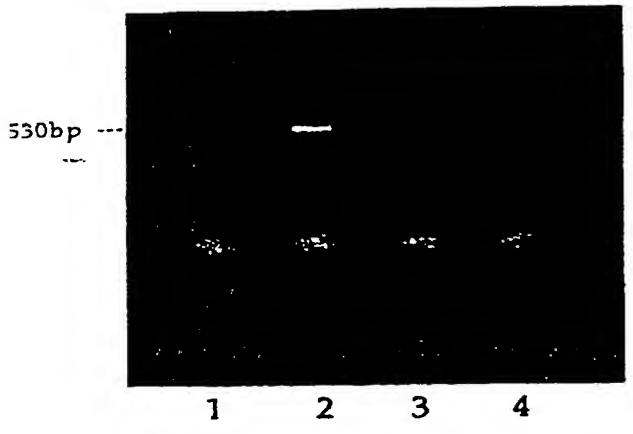

Fig. 3b. Loss of expression of IL-2 in tumours recovered from mice injected with clone 3 cells. The tumours were snap-frozen and examined for expression of the IL-2 gene using primers which recognize a 530 bp IL-2 gene fragment. Lane 1: mock rt-PCR (no cDNA added to the reaction mix); lane 2: rt-PCR from 10^2 cells of clone 3 maintained in culture; lanes 3 and 4: rt-PCR of cDNA recovered from the tumours which grew in the clone 3-injected mice.

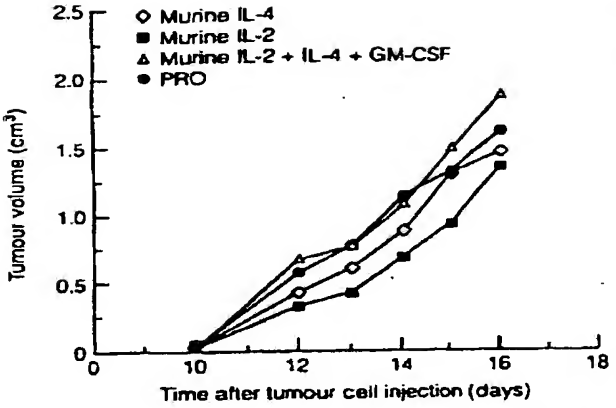

Fig. 4a. Effects of direct injection of DNA into established B16 tumours.

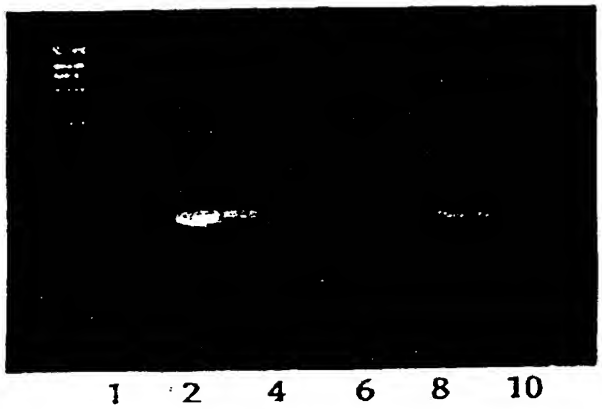

Fig. 4c. IL-4 expression following intratumoural injection of DNA. Primers to the murine IL-4 gene were used, which span the entire 450 bp murine IL-4 sequence. Lane 1: mock PCR; lanes 2–7: rt-PCR from 10^6, 10^5, 10^4, 10^3, 10^2 or 10 clone 28 cells in a total of 10^6 cells; lane 8: rt-PCR from a tumour injected with Tyr-IL-4 alone; lane 10: rt-PCR from a tumour injected with the cytokine-expressing plasmid combination; lane 11: rt-PCR from a tumour injected with PRO alone.

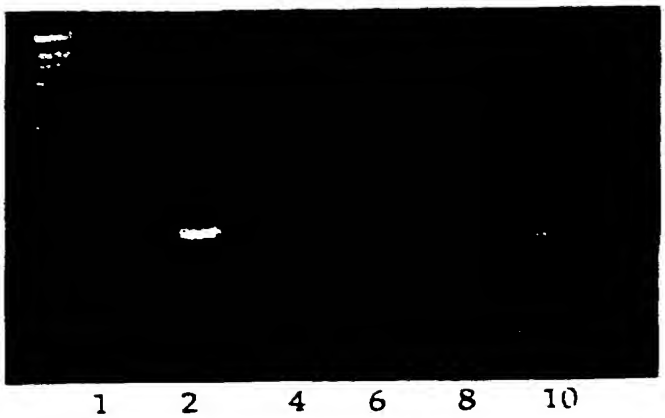

Fig. 4b. IL-2 expression following intratumoural injection of DNA. Primers were used which recognize the extreme 5′ and 3′ ends of the IL-2 gene (separated by 530 bp). Lane 1: mock PCR; lanes 2–8: rt PCR from 10^6, 10^5, 10^4, 10^3, 10^2, 10 or 0 clone 28 cells in a total of 10^6 cells; lane 9: rt-PCR from a tumour injected with Tyr-IL-2 alone; lane 10: rt-PCR from a tumour injected with the cytokine-expressing plasmid combination; lane 11: rt-PCR from a tumour injected with PRO alone. Similar results were generated from two separate tumours from each group.

of the tyrosinase promoter and the 3′ primer recognizes the 3′ end of the IL-2 gene. No signal was detected in the rt-PCR reaction from tumour samples, showing that the signals detected in Figs. 4b and 4c are due to RNA in the tumour samples and not to artefacts as a consequence of residual DNA.

Conclusions

We have demonstrated that the tyrosinase promoter directs tissue-specific expression of cytokine genes in melanoma cells in vitro, at levels sufficient to abrogate tumorigenicity when the cells are injected into immunocompetent syngeneic animals. This anti-tumour effect was consistently seen even in clones which produce only relatively small amounts of IL-2 (e.g. clone 28 and clone 34). However, loss of gene expression (either at the transcriptional or genomic level) was associated with restored tumorigenicity. Our results, here and elsewhere [5], show that the tyrosinase promoter is very effective in achieving high levels of recombinant gene expression in melanoma cells.

Several experiments in vivo have shown that the immune system can play a role in protection against malignancy [15,16]. This had led many groups to propose that tumour cells constitutively producing cytokines, such as IL-2 [13], IL-4 [17] and granulocyte-macrophage colony stimulating factor (GM-CSF) [18], could be used as 'cancer vaccines' [1]. Generally, such vaccination protocols have used tumour cells modified in vitro (either autologous [15] or allogeneic [19]) followed by their transfer back into the patient. However, these procedures usually require an appreciable period (4–6 weeks) of culturing of tumour cells to achieve efficient levels of gene transduction. This process may select cell variants which differ significantly in their antigenic profile from the parental population. Although improved viral vectors and infection protocols have recently been described for such ex vivo modifications [20], introduction of recombinant genes directly into malignant tumours in vivo could eliminate the need to establish cell lines from patients [21] or even from HLA-matched individuals [19]. This study reports an important step towards this goal by showing that direct injection of DNA encoding the Tyr-IL-2, Tyr-IL-4 or Tyr-M-CSF minigenes into established tumours in vivo leads to long-term expression (over 16 days) of the cytokines within the tumour deposit. The formal possibility that the IL-2, IL-4 or M-CSF mRNA detected by rt-PCR is contributed partly by infiltrating cells recruited to the cytokine-expressing tumours can-

not be excluded. The majority of the signal, however, is probably produced by expression of the injected minigenes, as injection of one-third as much DNA led to a similar reduction in intensity of the rt-PCR signal (Figs. 4b and 4c). Even if some of the observed expression of IL-2 or IL-4 is due to infiltrating immune cells (not seen in tumours injected with the control plasmid), the presence of such infiltrates may still relate to good anti-tumour immune response and prognosis [15, 22, 23]. The authors are currently investigating the different populations of infiltrating immune cells which are recruited to tumours injected with different cytokine cDNAs [24].

To date, a significant anti-tumour effect on the growth of the injected tumours using cytokine cDNAs has not been observed. This is not wholly unexpected. Although transfer of the IL-2 gene to B16 cells has been shown to abrogate tumorigenicity, both in this study and in others [13,18], a high percentage of the tumour cells (at least 50%) must express the gene at the time of injection for these effects to be observed [13]. The B16 melanoma is a rapidly growing, poorly immunogenic tumour whose rejection would require very high efficiency of gene transfer and rapid expression of the IL-2 gene. These results with rt-PCR and a reporter gene suggest only a low proportion of tumour cells (at most 10%) are expressing the injected genes 16 days after injection into the tumour mass [5]. Perhaps insufficient numbers of cells can be transduced quickly enough in this model to observe appreciable immune reactions, though other model systems using multiple injections of DNA have now been shown to respond positively to similar protocols [25].

Experiments to determine the optimal therapeutic gene, or combinations of genes, for the immunotherapy of malignant melanoma to be delivered by direct injection of DNA are underway. Several studies have suggested that other cytokines, such as GM-CSF [18], or immunomodulatory molecules such as B7 [26,27], may have tumour-protective effects in melanoma. One recent study has shown that injection of liposome-DNA complexes encoding a foreign MHC molecule generates a systemic anti-tumour immune response [25]. As a tissue-specific promoter was not used in these studies, expression of the construct occurred in normal as well as tumour tissue [25, 28]. While the full importance of restricting expression of the therapeutic gene to the tumour cells alone is unclear [25], it may be that inappropriate expression of such genes in normal tissues might trigger autoimmune and pathological changes. The prudence of trying to limit gene expression at the transcriptional level to tumour cells is emphasized by recent findings that intravenous delivery of DNA-liposome complexes leads to a widespread transduction of many different cell types in the body [29].

The experiments reported here show that direct injection of tissue-specific minigenes leads to intratumoural expression of potentially therapeutic genes. These early results provide a basis for developing a potentially simple and effective route for the eventual immunotherapy of malignant melanoma.

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Discussion

Question: Using your protocol, will you be able to detect who has been treated because they will be albino?
Professor Hart: There is a possibility that we will affect normal melanocytes, but that would be a minor consideration if we could do some good.

Question: You used reverse transcriptase polymerase chain reaction to define non-expression. How far down do you go? How many cycles?
Professor Hart: About 30–40 cycles would be routine.

Question: When you get to 60 cycles, you find all sorts of peculiar levels of expression of so-called tissue specific genes that can be a problem.
Professor Hart: These things are perhaps not as specific as I presented them, but we see about a 40–50 fold difference in expression between the melanocytic cells and other cell types.

Question: Is the incorporated DNA stable in the tumour cell? Also, if the tumour cell is lysed, is the DNA taken up by other cells?
Professor Hart: So far, we have achieved stability of expression up to 21 days after injection. Others have reported stability up to 90 days.

I do not know the answer to the second part of your question, but it is perfectly possible that cells will pass the DNA on.